

Mycobacterium leprae DNA in peripheral blood may indicate a bacilli migration route and high-risk for leprosy onset

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Abstract

Leprosy epidemiological studies have been restricted to *Mycobacterium leprae* DNA detection in nasal and oral mucosa samples with scarce literature on peripheral blood. We present the largest study applying quantitative real-time PCR (qPCR) for the detection of *M. leprae* DNA in peripheral blood samples of 200 untreated leprosy patients and 826 household contacts, with results associated with clinical and laboratory parameters. To detect *M. leprae* DNA a TaqMan qPCR assay targeting the *M. leprae* ML0024 genomic region was performed. The ML0024 qPCR in blood samples detected the presence of bacillus DNA in 22.0% (44/200) of the leprosy patients: 23.2% (16/69) in paucibacillary (PB), and 21.4% (28/131) in multibacillary (MB) patients. Overall positivity among contacts was 1.2% (10/826), with similar percentages regardless of whether the index case was PB or MB. After a follow-up period of 7 years, 26 contacts have developed leprosy. Comparing the results of healthy contacts with those that become ill, ML0024 qPCR positivity at the time of diagnosis of their index case represented an impressive 14.78-fold greater risk for leprosy onset (95% CI 3.6–60.8; $p < 0.0001$). In brief, contacts with positive PCR in blood at diagnosis of index cases are at higher risk of later leprosy onset and this marker might be combined with other prognostic markers for management of contacts, which requires further studies.

Keywords: Blood-borne pathogens, epidemiology, leprosy, *Mycobacterium leprae*, real-time PCR

Original Submission: 29 May 2013; **Revised Submission:** 20 July 2013; **Accepted:** 24 July 2013

Editor: E. Bottieau

Article published online: 28 August 2013

Clin Microbiol Infect 2014; **20**: 447–452

10.1111/1469-0691.12349

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Introduction

Leprosy is a complex disease caused by *Mycobacterium leprae*, an obligate intracellular pathogen with tropism for Schwann cells and cells of the mononuclear phagocyte system. The disease has a wide variety of clinical presentations which are classified into a spectrum of clinical forms according to the patient's immunological status; two polar forms, tuberculoid

(TT) and lepromatous (LL) leprosy, and three unstable borderline groups: borderline-tuberculoid (BT), borderline-borderline (BB) and borderline-lepromatous (BL) [1]. The tuberculoid side of the spectrum (TT and BT leprosy) is characterized by vigorous cell-mediated immune response, well-defined skin and nerve lesions, and few bacilli (paucibacillary leprosy, PB). Across the five-group system, the host presents gradual reduction of cell-mediated immune responses towards the lepromatous side of the spectrum (BL and LL), associated with increased bacillary load, several skin and nerve lesions, and high antibody titres (multibacillary leprosy, MB) [2].

Untreated MB patients are considered the main source of *M. leprae* transmission [3,4]. However, the number of MB patients is small and cannot represent the sole source of infection. Currently, the possibility of bacilli spread is not

restricted to leprosy patients, healthy carriers and individuals with subclinical infections, i.e. household contacts who carry *M. leprae* in their nasal [4–7] and/or oral mucosa [8], may play an important role in the disease chain of transmission.

Detection of *M. leprae* DNA by conventional PCR has achieved significant sensitivity and specificity when compared with conventional bacilli staining techniques [9–11]. However, quantitative real-time PCR (qPCR) has displayed even higher sensitivity and specificity in diagnosis of several parasites, and has been successfully applied to detect *M. leprae* DNA, mainly in skin smears and biopsy samples [8,12,13].

Clinical signs of leprosy are most commonly observed in the skin and peripheral nerves, but it is suggested that bacteraemia may occur at some point during disease progression [14]. The presence of *M. leprae* in peripheral blood has not been thoroughly investigated, although some reports have shown an infrequent presence of *M. leprae* in the blood of leprosy patients using both staining techniques [14] and conventional PCR [3]. At this time there are no publications on the qPCR detection of *M. leprae* DNA in peripheral blood samples.

It is necessary to define if besides indicating subclinical infection, *M. leprae* DNA in blood may represent a risk factor for leprosy development in these individuals. This study aims to evaluate the presence of *M. leprae* DNA in peripheral blood samples of leprosy patients and their household contacts.

Materials and Methods

Assays were performed in peripheral blood samples of 200 untreated leprosy patients and 826 household contacts attending the National Reference Centre for Sanitary Dermatology and Leprosy (CREDESH), MG, Brazil. Research protocol was approved by an independent research ethics committee and participants signed an informed consent form.

Study subjects

All leprosy patients were diagnosed by a committee of leprosy experts based on clinical and laboratory tests and were classified according to the Ridley–Jopling clinical forms five-group system (TT, BT, BB, BL, LL). For treatment purposes, all patients also received an operational classification into PB or MB [2].

Blood samples were collected from all patients at diagnosis and all underwent: Mitsuda test, an intradermal injection of 0.1 mL of a heat-killed *M. leprae* suspension of 6×10^7 bacilli/mL produced by the Lauro de Souza Lima Institute (ILSL-SP), with results observed 28 days later and stratified

according to the measurement in millimetres of the nodular epithelioid granuloma diameter: negative (0–3 mm), weakly positive (4–7 mm), positive (8–10 mm), strongly positive (>10 mm or of any size with ulceration); bacillary index in skin smears and bacillary index in skin lesion biopsies, with positive results when any bacilli were observed and negative when none were found; and anti-phenolic glycolipid-I (PGL-I) ELISA, an indirect ELISA to detect antibodies against the *M. leprae* native PGL-I molecule (donated by Dr John Spencer, Colorado State University, CO, USA), performed with a protocol standardized and previously described by our research group [15].

The CREDESH database holds records of 3113 contacts enrolled in the period from 2002 to 2012. A follow-up period of 7 years was established as sampling criterion and only contacts with a complete set of clinical and laboratory parameters were included, totalling 826 contacts.

These 826 household contacts were stratified according to clinical forms and operational classification of their index case. All the contacts were examined by specialist leprosy physicians for signs or symptoms that were suggestive of leprosy and all displayed normal dermato-neurological clinical examinations. Blood samples were collected from all contacts at diagnosis of their index case and they also underwent Mitsuda test and anti-PGL-I ELISA. Contacts were further stratified into two classes: affected (those who develop leprosy) and healthy (those without any sign or symptom). Table 1 summarizes these assessments of leprosy patients and household contacts.

DNA extraction and qPCR

DNA from 500 μ L peripheral blood was extracted with phenol and precipitated with ethanol. At the end, the DNA pellet was dried at room temperature and suspended in 50 μ L ultrapure water.

A region of 69 bp from a unique *M. leprae* genomic region (Gene: *ML0024*—GenBank Accession no. AL583917) was amplified, detected and quantified using a TaqMan primer/probe assay as previously described [8].

Statistical analysis

Differences between the groups were assessed by chi-squared or Student's *t*-test, when appropriate. The kappa test was applied to evaluate the agreement between the *ML0024* qPCR and other tests. Pearson's linear correlation test was employed to assess the correlation between *ML0024* qPCR and other tests. The OR and positive likelihood ratio were calculated to evaluate the performance of tests and estimate the probabilities for later leprosy onset. Statistical significance was set at $p < 0.05$.

TABLE 1. Laboratory tests results according to clinical forms and operational classification of leprosy patients and index cases of household contacts

Laboratory tests	Patients (n = 200)											
	Operational classification and clinical form											
	PB (n = 69)				MB (n = 131)							
	TT (n = 25)		BT (n = 44)		BT (n = 34)		BB (n = 34)		BL (n = 30)		LL (n = 33)	
	n	%	n	%	n	%	n	%	n	%	n	%
BI in skin biopsies positivity	1	4	8	18.2	19	55.9	23	67.7	28	93.3	32	97
BI in skin smears positivity	0	0	0	0	2	5.9	33	97.1	30	100	33	100
Mitsuda test positivity	23	92	40	90.9	15	44.1	1	2.9	0	0	0	0
Average Mitsuda (mm) ± SD	9.9 ± 2.5		6.6 ± 2.8		3.1 ± 3.2		0.5 ± 1.4		0 ± 0		0 ± 0	
Anti-PGL-I ELISA positivity	0	0	10	22.7	21	61.8	30	88.2	29	96.7	33	100
Average ELISA index ± SD	0.5 ± 0.3		0.8 ± 0.8		2.9 ± 3.4		4.9 ± 3.9		7.2 ± 5.1		7.8 ± 5.7	
ML0024 qPCR positivity	4	16	12	27.3	6	17.6	5	14.7	6	20	11	33.3
Laboratory tests	Household contacts (n = 826)											
	Operational classification and clinical form of index case											
	PB (n = 182)				MB (n = 644)							
	TT (n = 60)		BT (n = 122)		BT (n = 160)		BB (n = 144)		BL (n = 130)		LL (n = 210)	
	n	%	n	%	n	%	n	%	n	%	n	%
Mitsuda test positivity	54	90	111	91	141	88.1	135	93.7	115	88.5	184	87.6
Average Mitsuda (mm) ± SD	8.1 ± 4.4		7.0 ± 3.0		7.7 ± 3.5		7.7 ± 3.6		6.9 ± 3.3		6.9 ± 3.4	
Anti-PGL-I ELISA positivity	8	13.3	15	12.3	25	15.6	24	16.7	19	14.6	47	22.4
Average ELISA index ± SD	0.6 ± 0.4		0.6 ± 0.4		0.7 ± 0.5		0.8 ± 1.3		0.7 ± 0.6		1.2 ± 5.5	
ML0024 qPCR positivity	1	1.7	1	0.8	0	0	1	0.7	2	1.5	5	2.4

PB, paucibacillary; MB, multibacillary; TT, tuberculoid; BT, borderline tuberculoid; BB, borderline; BL, borderline lepromatous; LL, lepromatous leprosy; BI, bacterial index; SD, standard deviation; PGL-I, phenolic glycolipid I.
 For anti-PGL-I, considered positive result: ELISA index ≥ 1.1.

Results

The *M. leprae* qPCR in blood samples detected the presence of bacillary DNA in 22.0% (44/200) of leprosy patients: 23.2% (16/69) in PB patients, and 21.4% (28/131) in MB patients.

The ML0024 qPCR positivity assessed according to the patient's clinical form ranged from 16% (4/25) for TT to 33.3% (11/33) for LL patients (Table 1).

Among the 44 patients with positive ML0024 qPCR, 59% (26/44) were positive for anti-PGL-I ELISA, and 72% (32/44) had negative or weakly positive Mitsuda response.

The Kappa values obtained between the ML0024 qPCR and the tests mentioned were lower than 0.04, revealing lack of agreement.

The qPCR quantification of bacillary load in blood samples revealed values ranging from 870 up to 5.94×10^5 ML0024 DNA copies per mL of blood, which corresponds to TT and LL values, respectively. The average number of DNA copies did not show a direct relationship with the spectrum of clinical forms, nor with the operational classification (PB or MB).

Among household contacts, 1.2% (10/826) were positive for ML0024 qPCR in peripheral blood. DNA was detected in 1.1%

(2/182) of contacts of PB patients and in 1.2% (8/644) of contacts of MB patients (Table 1).

A high statistical significance was observed between the ML0024 qPCR positivity in blood of leprosy patients and household contacts ($p < 0.0001$).

All household contacts (10/10) with positive ML0024 qPCR also presented negative Mitsuda tests or weakly positive (0–7 mm) ($p = 0.0131$) and 40% (4/10) were positive for anti-PGL-I ELISA ($p = 0.1283$).

The contacts were followed up for a period of 7 years. In this period, 3.1% (26/826) developed leprosy, all of them were contacts of MB patients and 61.5% (16/26) were contacts of LL patients. Among these 26 contacts, ML0024 qPCR detected DNA from *M. leprae* in 11.5% (3/26), the ELISA test was positive in 57.7% (15/26), and Mitsuda test was negative or weakly positive in 84.6% (22/26).

The sensitivity and specificity parameters obtained for ML0024 qPCR in the blood of household contacts were 11.5% (3/26) and 99.1% (793/800), respectively. The ML0024 qPCR positivity among contacts presented an impressive OR of 14.78-fold towards leprosy development (95% CI 3.6–60.8; $p < 0.0001$), and positive likelihood ratio of 13.19 (95% CI: 3.6–48.1; $p < 0.0001$). The anti-PGL-I ELISA positivity presented

TABLE 2. Assessment of results for *ML0024* qPCR, anti-PGL-I ELISA, and Mitsuda tests based on the positivity at diagnosis of the index case observed in healthy household contacts ($n = 800$) in comparison with those contacts that were affected by leprosy ($n = 26$)

Test and type of contact	No. of positives	No. of negatives	Diagnostic parameters	OR (95% CI)	LR+ (95% CI)
<i>ML0024</i> qPCR					
Affected	3	23	Sensitivity 11.5%	14.78 (3.6–60.8)	13.19 (3.6–48.1)
Healthy	7	793	Specificity 99.1%		
Anti-PGL-I ELISA ^a					
Affected	15	11	Sensitivity 57.7%	7.51 (3.4–16.7)	3.75 (2.6–5.4)
Healthy	123	677	Specificity 84.6%		
Mitsuda test ^b					
Affected	4	22	Sensitivity 15.4%	0.23 (0.08–0.66)	0.34 (0.14–0.85)
Healthy	357	443	Specificity 55.4%		

LR+, positive likelihood ratio; PGL-I, phenolic glycolipid I.
^aFor anti-PGL-I, considered positive result: ELISA index ≥ 1.1
^bFor Mitsuda test, considered positive result: node > 7 mm.

7.51-fold higher risk of becoming ill (95% CI 3.4–16.7; $p < 0.0001$); whereas the positive Mitsuda test (> 7 mm) showed a 4.35-fold greater chance of protection (OR 0.23; 95% CI 0.08–0.66; $p = 0.0058$) (Table 2).

Discussion

The present study is the largest study to evaluate the presence of *M. leprae* DNA in peripheral blood samples of leprosy patients and household contacts. We have shown that the presence of *M. leprae* DNA in blood indicates a higher risk for leprosy development in contacts, which suggests a likely path of the infection process, after its passage through the mucosa of the upper respiratory tract and before the impairment of the peripheral nervous system. This infection process implicates a transient passage of bacilli through the bloodstream; almost one-quarter of patients presented positive PCR. However, the limitations of this study are the lack of multiple samples from the same individual collected at the same time and at different time-points, to demonstrate whether variability of bacterial DNA detection is due to sampling or to its microenvironmental requirements, which must be further investigated.

PCR positivity presented no correlation with clinical forms, anti-PGL-I ELISA, bacillary index, and Mitsuda test. Nevertheless, LL patients presented twice the frequency and higher bacterial load than observed in TT patients. It is debatable whether bacilli should not be expected in tuberculoid forms of the disease, as macrophages would kill and degrade the bacillus, including DNA, but *M. leprae* is an obligate intracellular pathogen that invades and multiplies within host mononuclear phagocytes, and immature monocytes can be reservoirs of fully viable DNA during *M. leprae* blood migration, a cellular event that seems to be required before the infection of Schwann cells and tissue macrophages.

The complete mechanism of infection of *M. leprae* is still unknown, but evidence of nasal and oral mucosa as entry sites have been provided [6–8,16]. Authors of the first seminal

publication that found *M. leprae* in the peripheral blood of LL patients have suggested that bacilli may be continuously present, but it may decrease during treatment [14]. Similarly, the detection of *M. leprae* DNA in nasal swabs of household contacts [4] reduced from 4% to 0% when the index case in each household was treated for 2 months, showing that effective MDT decreases repeat infection within the household.

Although the specificity of the PCR test in the blood was very high (99.1%), the sensitivity was low (11.5%), and no test alone would be worthwhile with such a low sensitivity, unless combined with other tests to bolster the leprosy diagnosis. It is relevant to emphasize that blood is not the best sample for the diagnostic detection of *M. leprae*, its regular temperature around 37°C does not constitute a suitable environment for the development and survival of *M. leprae*. The bacillus is presumably detected in the blood during the short period in which the infected cells migrate to the extremities of the body, where temperature ranges from 30 to 35°C, which favours *M. leprae* reproduction [17].

The presence of up to 10^5 viable leprosy bacilli/mL of blood both reflects and helps to explain the extreme widespread nature of *M. leprae* infection in patients with lepromatous leprosy [18]. The high titres of *M. leprae*-specific antibodies, although prevalent in LL leprosy patients, do not provide protection [19], and these factors may favour blood dissemination of the bacillus and its detection.

On the other hand, the specific immune cellular response observed among TT leprosy patients plays an important role against bacillary proliferation and dissemination, confining the bacillus to a few areas of the skin and/or nerves [17]. At the same time, PCR positivity observed in TT and BT patients might also imply an ongoing attack to the reticuloendothelial system and the recirculation of infected phagocytes into the bloodstream, which may account in part for the intermittent nature of leprosy bacteraemia [14].

Two studies have addressed the detection of *M. leprae* DNA in blood through conventional PCR. One of these

evaluated leprosy patients 8 years after MDT and observed 70.6% (12/17) positivity [20]. Having been conducted in an endemic area, the hypothesis of re-infection or relapse cannot be excluded, and a selection bias, due to the small number of patients evaluated, could not be ruled out. The second study evaluated 119 household contacts and observed 1.7% (2/119) positivity [9], which corroborates with 1.2% positivity observed in the present study, suggesting a similar PCR efficiency, especially considering that our sampling was eight times larger. The percentage of *M. leprae* DNA in peripheral blood samples of household contacts was 18 times lower than that among patients, similar to other studies that applied conventional PCR followed by a ^{32}P -labelled probe hybridization [9].

Recently, the *M. leprae*-specific repetitive sequence marker which targets an invariant region of the RLEP noncoding chromosomal element of dispersed repeats, has been shown to be more sensitive than other primer sets of unique alignment [11], most likely because its repetitive sequence is presented several times in the genome. Perhaps, a qPCR targeting RLEP sequence, which detects very low levels of DNA, would provide higher positivity rates in peripheral blood.

All PCR-positive contacts also presented negative or weakly positive Mitsuda test results, suggesting that besides the prognostic value, the immune cellular response cooperates in the early resistance against bacillary invasion of the mucosa.

The comparison of the presence of *M. leprae* DNA in the blood of contacts and the development of leprosy in this group showed an impressive 14.78-fold association between positive PCR and later disease onset. Besides that, the fact that all those who became ill were contacts of MB patients supports the theory that contacts of MB patients are at greater risk for the development of leprosy [21–23].

The small percentage of PCR positivity among contacts can disclose an important public health issue. Considering the latest official data of 219 075 new cases detected in the world [24] and an average of four household contacts per patient, this leads to an estimate of 876 300 household contacts of leprosy patients, and assuming the 1.2% PCR positivity in this group of individuals, it could be considered that 10 515 household contacts worldwide have *M. leprae* DNA in their bloodstream and are at greater risk of becoming ill.

Two publications [3,18] have shown that viable bacilli, potentially infective, were isolated from the peripheral blood of untreated leprosy patients, as judged by their capacity to multiply in the mouse footpad. However, because of the long-term test in mouse footpad with a restricted sensitivity, the detection of *M. leprae* mRNA through reverse transcription PCR may be a good option to evaluate the viability of blood-borne bacilli.

The carriage of bacilli in the blood of contacts in conjunction with greater chances for leprosy development in PCR-positive contacts are additional evidence of subclinical infection and those individuals without symptoms may behave as healthy carriers with a potential role in the chain of transmission of leprosy in endemic regions.

These findings have important implications for disease management and clinical practice. The concomitant use of tools to detect *M. leprae* DNA in the blood, anti-PGL-I circulating antibodies and the Mitsuda test, will allow us to identify high-risk individuals, justifying the chemoprophylaxis of close contacts of leprosy patients who fit the highest-risk categories in this study, as has been suggested elsewhere [25].

Acknowledgements

The authors are grateful to the entire team of the UFU National Reference Centre for Sanitary Dermatology and Leprosy, to Dr Maria Esther Salles Nogueira from Lauro de Souza Lima Institute for the donation of Mitsuda antigen, and to Dr John S. Spencer from the Department of Microbiology, Immunology and Pathology of the Biomedical Sciences and Veterinary Medicine College of the Colorado State University, CO, USA, for the donation of the PGL-I antigen for the ELISAs.

Financial Support

This study was supported with grants from: Foundation for Research Support of the State of Minas Gerais (FAPEMIG), Brazilian National Council for Scientific and Technological Development (CNPq), Brazilian Coordination for Improvement of Higher Education Personnel (CAPES), and by the National Fund for Health/Brazilian Ministry of Health.

Transparency Declaration

All authors declare no conflicts of interest.

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